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Reduced Polymorphism in the Kelch Propeller Domain in *Plasmodium vivax* Isolates from Cambodia

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Polymorphism in the ortholog gene of the *Plasmodium falciparum* K13 gene was investigated in *Plasmodium vivax* isolates collected in Cambodia. All of them were Sal-1 wild-type alleles except two (2/284, 0.7%), and *P. vivax* K12 polymorphism was reduced compared to that of the *P. falciparum* K13 gene. Both mutant allele isolates had the same nonsynonymous mutation at codon 552 (V552I) and were from Ratanak Kiri province. These preliminary data should encourage additional studies for associating artemisinin or chloroquine resistance and K12 polymorphism.

In areas in which malaria is endemic and *Plasmodium falciparum* and *Plasmodium vivax* are present, such as in Southeast Asia and Pacific Oceania, both species share the same vectors and human hosts, either successively or concomitantly (mixed infections) (1, 2). These two species, in this context, often undergo similar mutation-driven evolution and natural selection. This includes nucleotide substitution, gene duplication/deletion, chromosomal change, and genome duplication (3). In terms of drug resistance, regardless of the fundamental biological differences between the two *Plasmodium* species, it is well known that antimalarial drug pressure induces a strong selection of resistant parasites for both of these parasite populations. For instance, sequencing of the *dhfr* gene in *P. vivax* isolates collected in areas where sulfadoxine-pyrimethamine was used to treat falciparum malaria and the alignment of these alleles with the *P. falciparum dhfr* gene have clearly demonstrated that mutations in codons 57, 58, 61, 117, and 173 were involved in pyrimethamine resistance and corresponded to the codons 51, 59, 108, and 164 found in *P. falciparum* pyrimethamine-resistant strains (4). More recently, we observed high frequencies of *P. falciparum* and *P. vivax* isolates with increased *mdr-1* copy numbers in areas where mefloquine has been extensively used as the first-line treatment in falciparum-uncomplicated malaria, while in areas where mefloquine has never been used, *P. falciparum* and *P. vivax* isolates with increased *mdr-1* copy numbers are rare (5). These studies clearly show that antimalarial drugs used to treat falciparum malaria have a significant impact on sympatric *Plasmodium* species, such as *P. vivax*.

Since 2001, artemisinin combination therapies (ACTs) have been recommended as first-line treatment in the national treatment guidelines of most countries in which malaria is endemic. In 2008, the emergence of artemisinin-resistant *P. falciparum* parasites was observed in Southeast Asia (6–15). Recent molecular and biological studies showed that artemisinin resistance was associated with *P. falciparum* early ring stages and mutations in the *PF3D7_1343700* kelch propeller domain (K13-propeller) (8, 14, 15). To date, although the role of the *P. falciparum* K13 protein remains unknown, two pieces of evidence suggest that it is involved in the cellular response to oxidative stress (8): (i) its homology to the KEAP1 human protein, which is involved in cell adaptation to oxidative stress (16), and (ii) the pro-oxidant activity of artemisinin derivatives (17).

In Cambodia, antimalarial drug resistance is a major concern. Since 2001, ACTs (artesunate plus mefloquine and, later, dihydroartemisinin plus piperaquine) have been used as first-line treatment for falciparum malaria. For vivax malaria, chloroquine, a drug that also induces oxidative stress (18), was abandoned in 2012 and replaced by dihydroartemisinin plus piperaquine. This change was based on data from clinical therapeutic efficacy studies (day 28 follow-up, PCR-uncorrected WHO protocol) that showed proportions of treatment failure ranging from 0% to 17.4% (in Ratanak Kiri province, 2010) following a chloroquine regimen, while in the same areas, dihydroartemisinin plus piperaquine was 100% effective (19). It is worth noting that chloroquine resistance in this area was not fully confirmed due to the lack of blood concentration measures and genotyping data (between the isolates on day 0 and those on the day of recrudescence). Moreover, as no reliable molecular marker associated with *P. vivax* chloroquine resistance has been identified yet, it remains difficult to assess from clinical studies the antimalarial drug resistance of *P. vivax* (due to confounding factors, such as relapse, reinfection, or recrudescence).

As *P. vivax* appears to be highly sensitive to oxidative stress (its tropism in reticulocytes probably reflects this sensitivity), investigations of the polymorphism in the orthologous *P. vivax* gene of the *PF3D7_1343700* kelch propeller domain gene were performed, and the main objective was to assess the proportion of parasites with mutant alleles in our recent collection of venous samples collected from symptomatic individuals with *P. vivax* malaria from 2011 to 2013 in 6 different health centers.

The alignment of the *PF3D7_1343700* sequence with the *P. vivax* reference Sal-1 genome identified an orthologous gene located on chromosome 12 (*PVX_083080*, named K12 here). A nested PCR approach was designed to amplify the K12 propeller domain (from

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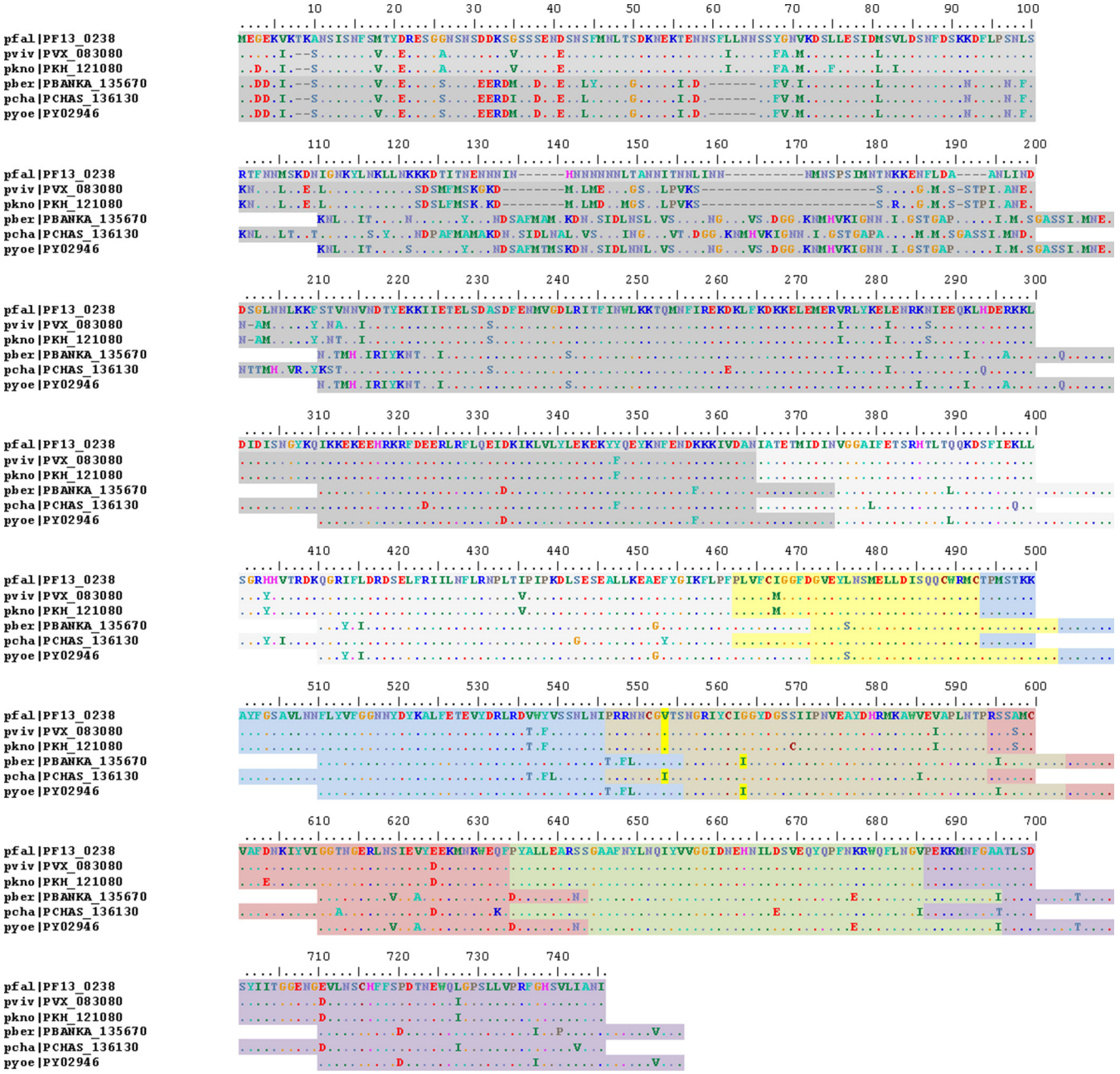

FIG 1 Sequence alignment of the propeller domain of the *P. vivax* K12 with the *P. falciparum* K13 protein. The V552I mutation detected in two *P. vivax* isolates is highlighted in yellow. Dark gray, *Plasmodium* and *Apicomplexa* specific; light gray, BTB/POZ domain; yellow, blue, brown, red, green, and purple, six individual kelch domains; pfal, *P. falciparum*; pviv, *P. vivax*; pkno, *Plasmodium knowlesi*; pber, *Plasmodium berghei*; pcha, *Plasmodium chabaudi*; pyoe, *Plasmodium yoelii*.

codons 370 to 702; Fig. 1) using the primers F-K12_P1 (5′-ATCCAACAGCATTTCCAACT-3′) and R-K12_P1 (5′-CAATTAAAACGGAATGTCCA-3′) for the outer PCR and F-K12_P2 (5′-ACCACGTGACGAGGGATAAG-3′) and R-K12_P2 (5′-AAAACGGAATGTCCAAATCG-3′) for the inner PCR. Briefly, parasite DNA was extracted from whole blood using the QIAamp DNA blood mini-kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The parasite species was confirmed by real-time PCR, as described by Canier et al. (20). The first round of PCR amplification was performed with a 20-μl reaction mixture containing 5 μl DNA, 0.25 μM each primer, 2.5 mM $MgCl_2$, and 0.25 μl HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) under the following conditions: 95°C for 15 min, followed by 20 cycles at 95°C for 30 s, 58°C for 60 s, and 72°C for 130 s, and a final extension at 72°C for 10 min. Nested PCR amplifications were performed in a 20-μl reaction buffer with 5 μl of the primary PCR products (10-fold diluted), 0.25 μM each primer, 2.5 mM $MgCl_2$, and 0.25 μl HOT FIREPol DNA polymerase under the following conditions: 95°C for 15 min, followed by 35 cycles at 95°C for 30 s, 62°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. Sequencing of the PCR products was performed by Macrogen (Seoul, South Korea), and sequences were

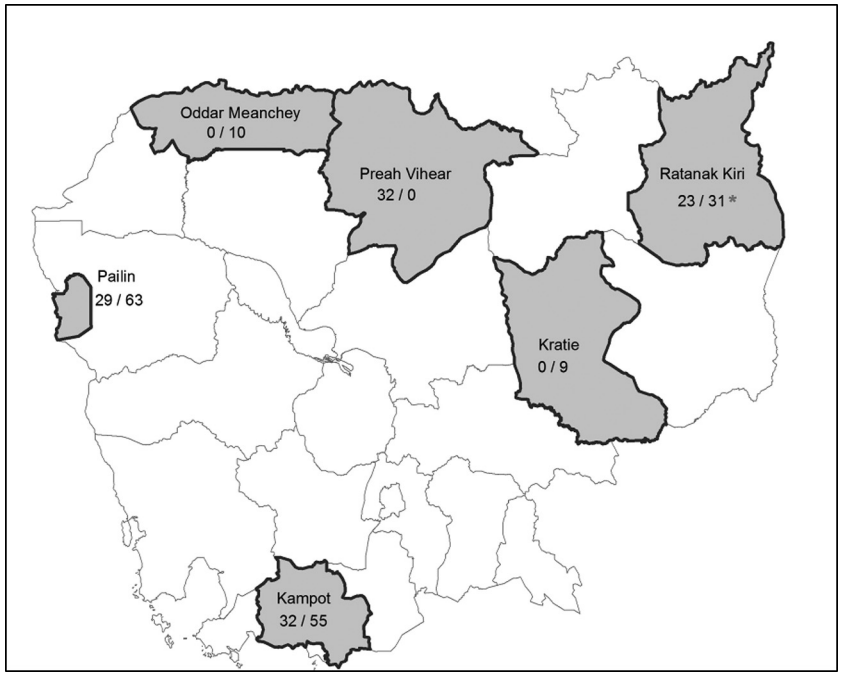

FIG 2 Numbers and sampling locations of Cambodian *P. vivax* isolates selected for K12 sequencing. The 6 provinces where the samples were collected are highlighted in gray. The two numbers under each province name represent the numbers of isolates sequenced in 2011/2013. The two isolates carrying the V552I mutation were collected in Ratanak Kiri province (asterisk).

aligned against the Sal-1 reference sequence (*PVX_083080*) using MEGA6. All alignments were manually verified.

A total of 284 Cambodian *P. vivax* isolates were selected and sequenced (Fig. 2). All but two (0.7%) sequences were interpretable and were Sal-1 wild-type alleles. The two isolates, collected in 2013 in Ratanak Kiri province in eastern Cambodia, were carrying the same nonsynonymous mutation at codon 552 (V552I) and showed a reduced polymorphism in the *P. vivax* kelch propeller domain (Fig. 1) (18).

The proportions of *P. vivax* K12 and *P. falciparum* K13 mutant alleles in samples collected during the same period showed a significantly higher frequency ($P < 10^{-6}$) in Pailin province (western Cambodia) for *P. falciparum* K13 (95.0% [95% confidence interval (CI), 88.0% to 99.0%]) than for *P. vivax* K12 (0% [95% CI, 0% to 4.0%]), while the frequencies were similar ($P = 0.66$) in Ratanak Kiri province (6.0% [95% CI, 1.0% to 19.0%] for *P. falciparum* K13 versus 3.7% [95% CI, 0.4% to 13.4%] for the *P. vivax* K12). These data seem to indicate that artemisinin drug pressure in western Cambodia is not selecting the *P. vivax* K12 mutation, contrary to what we have observed with *P. falciparum* K13. However, as we have never observed artemisinin-resistant *P. vivax* parasites in this area, we cannot rule out its role in artemisinin resistance.

Regarding chloroquine resistance, the two K12 mutant alleles were observed in Ratanak Kiri province, where chloroquine resistance is quite frequent. As no resistant phenotype was available for these two isolates (no *ex vivo* drug susceptibility assay and no *in vivo* follow-up of the patients), no association between polymorphism in the K12 propeller domain and chloroquine resistance can be assessed.

Given the high degree of conservation of the protein sequence between different *Plasmodium* species, it is intriguing to detect those two mutants. However, it must be pointed out that (i) the replacement of the valine at codon 552 by an isoleucine is not a major protein change (both are nonpolar branched-chain amino acids), and (ii) isoleucine is found at this position in 3 mouse parasite Kelch sequences (Fig. 1).

In conclusion, although we were limited by a lack of clinical and *in vitro* data, our study presents, for the first time, polymorphism in the orthologous *P. vivax* gene of the *PF3D7_1343700* kelch propeller domain gene compared to that of *P. falciparum* K13. These preliminary data should encourage additional studies on this gene that aim to associate artemisinin or chloroquine resistance based on clinical or *in vitro* phenotypes to K12 propeller domain protein polymorphism, especially in Indonesia, East Timor, Papua New Guinea, and South America (Guyana, Peru, and Brazil), where chloroquine resistance is frequent (21).

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We declare no conflicts of interest.

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